Sensitive and specific detection of ligands using engineered riboswitches

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\textbf{A R T I C L E   I N F O}

\textbf{Keywords:}
Riboswitch
Biosensor
Specificity
Sensitivity
In vitro
Selection

\textbf{A B S T R A C T}

Riboswitches are RNA elements found in non-coding regions of messenger RNAs that regulate gene expression through a ligand-triggered conformational change. Riboswitches typically bind tightly and specifically to their ligands, so they have the potential to serve as highly effective sensors in vitro. In B. subtilis and other gram-positive bacteria, purine nucleotide synthesis is regulated by riboswitches that bind to guanine. We modified the xpt-pbuX guanine riboswitch for use in a fluorescence quenching assay that allowed us to specifically detect and quantify guanine in vitro. Using this assay, we reproducibly detected as little as 5 nM guanine. We then produced sensors for 2′-deoxyguanosine and cyclic diguanylate (c-diGMP) by appending the P1 stem of the guanine riboswitch to the ligand-binding domains of a 2′-deoxyguanosine riboswitch and a c-diGMP riboswitch. These hybrid sensors could detect 15 nM 2′-deoxyguanosine and 3 nM c-diGMP, respectively. Each sensor retained the ligand specificity of its corresponding natural riboswitch. In order to extend the utility of our approach, we developed a strategy for the in vitro selection of sensors with novel ligand specificity. Here we report a proof-of-principle experiment that demonstrated the feasibility of our selection strategy.

\section{Introduction}

Riboswitches are regulatory elements, found in the non-coding regions of messenger RNAs, that control gene expression through the direct sensing of signaling molecules (Serganov and Nudler, 2013). Binding of a specific ligand to a riboswitch stabilizes one of two alternative conformations resulting in either an increase (“ON” switch) or decrease (“OFF” switch) in the level of gene expression. Riboswitches can control transcription, translation, splicing, or RNA stability. To date, riboswitches have been discovered that respond to ions, purines and purine derivatives, enzyme cofactors, and amino acids. Since the discovery of riboswitches, there has been an explosion of interest in exploiting their high affinity and specificity for their ligands to develop biosensors for monitoring the concentration of compounds in living cells or in solution (Fowler et al., 2010; Fowler et al., 2013; You et al., 2015; Su et al., 2016). There is also great interest in using riboswitches as novel gene regulatory modules in synthetic biology (Topp and Gallivan, 2010; Chappell et al., 2013; Groher and Suess, 2014; Etzel and Morl, 2017; Hallberg et al., 2017).

Riboswitches are modular. They consist of a ligand-binding domain (“aptamer domain”) and an “expression platform” that changes conformation in response to ligand binding. Sometimes, domains from two different riboswitches can be swapped to generate new synthetic riboswitches (Ceres et al., 2013aa,b; Litke et al., 2016; Rossmanith and Narberhaus, 2016). In addition, aptamers produced by in vitro selection have been successfully coupled to expression platforms to produce artificial riboswitches (also called “signaling aptamers”) or to ribozymes to produce ligand-regulated allosteric “aptazymes” (Soukup and Breaker, 1999; Robertson and Ellington, 2000; Sharma et al., 2008). These constructs have been used to monitor ligand concentration in vivo and in vitro or to regulate gene expression in response to novel ligands.

We would like to produce new riboswitches with altered ligand specificity for use as biosensors. Producing highly effective biosensors through rational design is a challenging problem. Two examples from the literature are particularly relevant to our work. The purpose of these two experiments was not to produce effective sensors but to reveal the key elements that determine ligand specificity. Nonetheless, they illustrate the difficulty of using a rational design approach to
engineer biosensors with novel ligand specificity. C74 in the aptamer domain of the guanine riboswitch forms a Watson-Crick base pair with the guanine ligand (Serganov et al., 2004). When Gilbert et al. changed this C to U, the ligand preference changed from guanine to adenine but the mutant aptamer domain bound to adenine poorly compared to naturally-occurring adenine riboswitches (Gilbert et al., 2006). Adenine riboswitches have a U at position 74, but their sequences differ from that of the guanine riboswitch at many additional sites (Serganov et al., 2004). All of these differences are required to make them effective adenine sensors. Edwards and Batey gradually replaced sequences in the aptamer domain of the xpt-pbuX guanine riboswitch with the corresponding sequences from a 2'-deoxyguanosine riboswitch (Edwards and Batey, 2009). Only after extensive substitutions was a hybrid aptamer domain produced that could bind to 2'-deoxyguanosine with an affinity and specificity similar to the natural 2'-deoxyguanosine riboswitch.

Given the difficulties associated with rational design, it would be very useful to have a simple in vitro selection strategy for isolating riboswitches with novel ligand specificity. Traditional aptamers produced by in vitro selection are selected only for their ability to bind to a specific ligand. Thus, to produce an effective sensor by coupling a selected aptamer to an expression platform or to a ribozyme often requires extensive re-engineering and optimization (Ceres et al., 2013a,b; Soukup and Breaker, 1999; Robertson and Ellington, 2000). We and others have reported in vitro selection systems that directly select “signaling” aptamers that not only bind to a ligand but also signal the presence of the ligand by undergoing a specific conformational change (Nutu and Li, 2005; Morse, 2007; Rajendran and Ellington, 2008; Vandenbergel and Morse, 2009). Here we exploited the modular nature of naturally-occurring riboswitches to produce highly sensitive and specific sensors. We then used our sensor design and our previous approach for producing signaling aptamers as the bases for a selection strategy with the potential to directly select signaling aptamers with novel ligand specificity. We report a proof-of-principle experiment demonstrating the feasibility of our selection strategy.

2. Materials and methods

2.1. Fluorescence measurements

All measurements of fluorescence intensity were performed with a Modular fluorometer (Turner Biosystems) in raw fluorescence mode using the blue fluorescence optical kit. Measurements were reported in “fluorescence standard units (FSU)”. Sequences of other oligonucleotides tested for use in the fluorescence quenching assay are given in Fig. 3.

2.2. Oligonucleotides

Oligonucleotides were obtained from Integrated DNA Technologies, Inc. (IDT).

DM024 (GGTATAATAGGAACACTCTataatCCGGTGAGTGGCAC GCAagtttctaccGGGCACCGTAAATGTCCgactATGGGTGAGCAATGGA). Encodes c-diGMP-guanine hybrid riboswitch. Upper case bases are from DM024. Lower case bases are from a c-diGMP riboswitch found in Vibrio cholera (Ve2) (Sudarsan et al., 2008). We removed one nucleotide from each of the priming sites so we used two new primers for PCR (DM025S and DM026S below).

DM025S (GATAATACGCTACTAGTAGTAAATGGA). Upstream primer for amplifying JL002. It is identical to DM025 but is one nucleotide shorter.

DM026S (TCCATTGCTGCA). Downstream primer for amplifying JL002. It is identical to DM026 but is one nucleotide shorter. This oligo was HPLC purified.

2'T-1 (AGGGGGCGAAGTGC). Corresponds to the sequence of the 5' half of the terminator from the xpt-pbuX guanine riboswitch (Serganov et al., 2004).

3'T-1 (GGAGGGCGGATGCT). Corresponds to the sequence of the 3' half of the terminator from the xpt-pbuX guanine riboswitch (Serganov et al., 2004).

2'T-1 labeled with quencher (SIABFQ-CATTGCTCAACC). Variant of 5'T-1 chosen for use in fluorescence quenching assay. SIABFQ is the Iowa Black fluorescence quencher attached to the 5' end.

3'T-1 labeled with biotin (SBIotinTGG-CATTGCTCAACC). Biotin is attached to the 5' end of 2'T-1 via a 16 atom linker.

3'T-3 (GAGTGAGCAATG). Variant of 3'T-1 chosen for use in the fluorescence quenching assay.

Sequences of other oligonucleotides tested for use in the fluorescence quenching assay are given in Fig. 3.

2.3. Synthesis, labeling, purification, and quantification of RNA

Transcription templates were prepared by amplifying DM024, JL001, or JL002. Primers for amplifying DM024 and JL001 were DM025 and DM026. Primers for amplifying JL002 were DM025S and DM026S. Reaction contained: 5 μL (5 ng) oligonucleotide; 12 μL (240 pmol) of each primer; and 200 μL PCR supermix (Invitrogen). Reactions were divided into 4 equal aliquots and PCR was performed for 25 cycles. Each cycle consisted of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s. PCR products were pooled, diluted to 400 μL with water, extracted once with phenol:chloroform:isoamyl alcohol (25:24:1 v/v), ethanol precipitated, and dissolved in 30 μL water. The upstream PCR primers (DM025 and DM025S) included a T7 RNA polymerase promoter at their 5' ends allowing the PCR products to be used as templates for in vitro transcription. In vitro transcription was performed with the TranscribeAid T7 High Yield Transcription Kit (Thermo Scientific). Reactions contained: 30 μL (~600 ng) PCR product; 85 μL water; 64 μL 5× buffer; 116 μL 25 mM NaCl; 25 mM MgCl2; 25 μL enzyme solution. Reactions were split into 2 equal aliquots and incubated for 6 h at 40°C. 2 μL of 1 U/μL DNase was added to each tube and incubation was continued for another 15 min. After incubation, precipitates formed (likely pyrophosphate). The precipitates were pelleted by spinning for 3 min at top speed in a microcentrifuge, and the supernatants were transferred to new tubes. The samples were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1 v/v). Each reaction was split into 2 equal aliquots. Each aliquot was diluted to 400 μL with water and the RNA was precipitated. Each pellet was dissolved in 100 μL of water and two aliquots of 50 μL were each passed through a chromaspin-10 gel filtration spin column (Clontech). Each aliquot was diluted to 400 μL with water and precipitated and the pellets were dissolved in 100 μL of water. The aliquots were combined and the concentration of the RNA was determined by absorbance at 260 nm. Each RNA was labeled by oxidizing the 3' ends with sodium periodate (Sigma) and reacting the oxidized RNA with fluorescein-5-thiosemicarbazide (Sigma). Oxidation reactions contained: 144 μL (150 μg) RNA; 18 μL 200 mM NaCH3COO
pH 5.5; and 18 μL 200 mM NaIO₄ (0.5 g/10 mL; made fresh). The reactions were incubated for 1 h at room temperature in the dark. Excess NaIO₄ was consumed by adding 180 μL of 2% ethylene glycol and incubating for 10 min at room temperature in the dark. Each reaction was diluted to 800 μL with water, divided into two aliquots of 400 μL and the RNA was precipitated. The pellets were dissolved in 400 μL of water and the RNA was precipitated again and each pellet was dissolved in 97.5 μL of water and combined. Labeling reactions contained 195 μL of water, divided into two aliquots of 400 μL with 7.5 μL 100 mM fluorescein-5-thiosemicarbazide dissolved in dimethylformamide (Sigma). Reactions were incubated for 1 h at room temperature in the dark. The reactions were diluted to 400 μL with water and precipitated. Both labeled and unlabeled RNAs were purified from an 8% polyacrylamide gel by electroelution using D-tube dialysis tubes (Novagen). Electroeluted RNAs were precipitated and dissolved in 100 μL water. The concentration of each RNA was determined by absorbance at 260 nm. An aliquot of each labeled and gel-purified RNA was diluted 200-fold and the fluorescence intensity was measured. Fluorescence intensity was typically about 1.5 × 10⁵ FSU per pmol of RNA.

2.4. Electrophoretic mobility shift assays (EMSA)

To identify DNA oligonucleotides that could efficiently form a stable duplex with xpt RNA (1–91), we purchased 9 oligonucleotides (5’-T-2 through 5’-T-10) predicted to have a range of melting temperatures (Tₘ) when annealed to xpt RNA (1–91). 10 μL annealing reactions contained 0.1 μmol of fluorescein-labeled RNA, 0, 0.1, 1, or 10 μmol of oligonucleotide, and assay buffer (100 mM KCl, 50 mM Tris-HCl, pH 7.4). The mixtures were heated at 65 °C for 3 min and cooled to room temperature. To separate duplexes from single stranded nucleic acids, each annealing reaction was run on an 8% non-denaturing polyacrylamide gel at room temperature. The RNA-DNA duplexes and single-stranded RNAs were visualized on a UV trans-illuminator.

To identify DNA oligonucleotides that could efficiently compete with xpt RNA (1–91) for pairing with 5’T-7 in the presence of guanine, we purchased 5 oligonucleotides (3’T-2 through 3’T-6) predicted to have a range of melting temperatures when annealed to 5’T-7 and performed strand-exchange reactions. After preparing 10 pmol of the xpt RNA (1–91)/5’T-7 duplex as described above, 20 pmol of competing oligonucleotide in assay buffer, and 0, 20, or 40 pmol of guanine in assay buffer were added to a total volume of 15 μL. The reaction products were separated and visualized as described above for the annealing reaction. A successful strand-exchange reaction resulted in the release of single-stranded xpt RNA (1–91) from the xpt RNA (1–91)/5’T-7 duplex. After testing a variety of reaction temperatures and incubation times, we found that incubating the reaction overnight (∼18 h) at 4 °C gave the best signal-to-background ratio.
2.5. Fluorescence quenching assay

The following ligands were purchased from Sigma: guanine, adenine, hypoxanthine, guanosine, 2'-deoxyguanosine, 3'-deoxyadenosine, 2'-guanosine monophosphate (2'-GMP), 3'-guanosine monophosphate (3'-GMP), 3',5'-cyclic diguanylate (c-diGMP), 3',5'-cyclic diadenylate (c-diAMP), and 2',5'-3',5'-cyclic guanosine monophosphate-adenosine monophosphate (c-GAMP). Reactions (70 μL total volume) contained: 25 pmol unlabeled riboswitch RNA; 10 pmol riboswitch RNA labeled at 3'-deoxyguanosine-guanine hybrid and the c-diGMP-guanine hybrid riboswitches also included 2 mM MgCl₂. Prior to adding 3'-guanosine monophosphate, 3'-deoxyadenosine, 3'-deoxyguanosine, 3'-deoxyadenosine-guanine monophosphate-adenosine monophosphate (c-GAMP), and 3'-deoxyguanosine-guanine hybrid riboswitch RNA by heating at 65 °C for 3 min in assay buffer and cooling to room temperature. The annealing reaction and a mix containing 3'-T-3, and assay buffer (including MgCl₂ when needed) were placed on ice. After 5 min on ice, the mix was added to the annealing reaction and incubated at 4 °C for ~18 h. After incubation, the fluorescence intensity was measured at 4 °C using assay buffer as the blank. A one-tailed T-test assuming equal variance was used to determine if signals were greater than background.

2.6. Preparation of initial partially randomized RNA pool for selection

An oligonucleotide pool containing a large number of variants of the guanine riboswitch was synthesized by IDT (see oligonucleotide DM024 in Section 2.2). Each of the lower case bases shown in the DM024 sequence was 27% randomized. For example, consider a randomized position that is shown as an A in DM024. Of the oligonucleotides tested for their ability to compete with xpt RNA (1–91) for pairing with 5'T-7. The sequence of 3'T-1 is the same as the 3' half of the terminator in the ligand-bound form of the guanine riboswitch. Bases that are not complementary to xpt RNA (1–91) are in lower case. Asterisks indicate oligos that could form a stable duplex with xpt RNA (1–91) when present in stoichiometric amounts. 5'T-7 (indicated by **) was chosen for use in the strand-exchange reaction. (B) Oligonucleotides tested for their ability to compete with xpt RNA (1–91) for pairing with 5'T-7. The sequence of 3'T-1 is the same as the 3' half of the terminator in the ligand-bound form of the guanine riboswitch. Bases that are not complementary to 5'T-7 are in lower case. 3'T-3 (indicated by **) was chosen for use in the strand-exchange reaction. (C) Strand-exchange reaction. The positions of fluorescein-labeled xpt RNA (1–91) (lane 1) and the duplex formed with 5'T-7 are indicated on the left side of the non-denaturing gel. When strand-exchange occurred, the duplex was converted into free xpt RNA (1–91). Very little strand-exchange occurred in the absence of guanine and 3'T (lanes 2) or in the presence of 3'T alone (lane 3). Strand-exchange occurred in the presence of 3'T plus 20 pmol of guanine (lane 4) and neared completion in the presence of 3'T and 40 pmol of guanine (lane 5).
randomized pool, 73% have an A, 9% have a G, 9% have a C, and 9% have a T at this position. The transcription template was made by amplifying the randomized oligonucleotide pool using DM025 and DM026 as primers. A total of 40 pmol (∼2 × 10^{13} molecules) of the randomized oligonucleotide pool was amplified in 30 independent reactions (50 μL each). The randomized RNA pool was synthesized, labeled, purified, and quantified as described above.

2.7. In vitro selection procedure

The selection strategy is illustrated in Fig. 9A. 200 pmol of biotinylated 5′T-7 was attached to 2 mg of streptavidin-coated magnetic beads (Dynabeads M-270 streptavidin from Invitrogen) according to manufacturer’s instructions. The beads were captured with a magnetic stand (Promega) and the liquid was removed. 180 pmol of fluorescein-labeled randomized RNA in 200 μL of hybridization buffer (50 mM Tris pH 7.4, 500 mM NaCl) was added to the beads and the mixture was rotated overnight at 25 °C to allow the RNA to anneal to immobilized 5′T-7. After RNA binding, unbound RNA was removed as follows: beads were rinsed 4 times quickly at 25 °C, twice for 30 min at 25 °C, and once for 30 min at 4 °C with 400 μL of assay buffer. The mixture was continuously rotated during each 30 min incubation. Beads were captured between each rinse and the liquid was discarded. Bound RNA was eluted for various times by rotating at 4 °C in 200 μL assay buffer.

Fig. 5. Predicted secondary structures of the guanine, 2′-deoxyguanosine, and hybrid riboswitches. The solid box indicates the P1 stem and flanking nucleotides 1–13 and 82–91 (dotted lines) of the guanine riboswitch. The dotted box indicates the ligand-binding domain of the 2′-deoxyguanosine riboswitch. The structures of guanine and 2′-deoxyguanosine are shown.

Fig. 6. Sensitivity and specificity of the 2′-deoxyguanosine hybrid sensor. (A) Fluorescence intensity as a function of 2′-deoxyguanosine concentration. The sensor was saturated at ∼1 μM 2′-deoxyguanosine. (B) Enlargement of the boxed region of the graph in (A) showing that the sensor can reproducibly detect as little as 15 nM 2′-deoxyguanosine. Fluorescence values are the averages of 3 independent reactions. Error bars are standard deviations. The asterisk indicates that the signal at 15 nM 2′-deoxyguanosine is significantly higher than background (P = 0.005). (C) The sensor retains the specificity of the natural 2′-deoxyguanosine riboswitch. The signal produced by 1 μM 2′-deoxyguanosine was compared to that produced by 1 μM and 50 μM guanosine, 1 μM and 50 μM 2′-deoxyadenosine, or 1 μM and 5 μM guanine. Fluorescence values are the averages of 3 independent reactions. Error bars are standard deviations. Asterisks indicate signals that were significantly higher than background (P < 0.05).
containing 5 μM guanine and the eluted RNA was collected. RNA that remained bound to the beads was removed by heating 3 times in 600 μL of hybridization buffer for 3 min at 55 °C. The RNA removed by each round of heating was collected in a single tube. The amount of RNA that eluted with ligand and the amount of RNA removed from the beads by heating was determined by measuring the fluorescence intensity of a small aliquot of each of the two fractions. The amount of RNA that bound to the 5'T-7 beads was calculated as the amount of RNA eluted with ligand plus the amount of RNA removed by heating. The elution efficiency was calculated as the percentage of bound RNA eluted with ligand. The level of background elution was determined in control experiments in which the RNA was eluted with assay buffer containing ligand plus the amount of RNA removed by heating. The elution with ligand and the amount of RNA removed from the beads by heating. The elution efficiency was calculated as the percentage of bound RNA eluted with ligand. The level of background elution was determined in control experiments in which the RNA was eluted with assay buffer. We found that the highest signal-to-background ratio was achieved when the RNA was eluted for 6 h. The eluted RNA was precipitated and dissolved in 19.2 μL of water. The RNA was amplified by reverse transcription and PCR followed by transcription. Reverse transcription reactions contained: 19.2 μL RNA; 2 μL DM026 (50 pmol); 1.6 μL 10 mM dNTP; 6.4 μL 5 × buffer (supplied with enzyme); 1.6 μL 100 mM DTT; and 1.2 μL (300 U) MLV reverse transcriptase (Invitrogen). The reactions were incubated for 1 h at 42 °C. PCRs contained: 32 μL cDNA (from reverse transcription); 16 μL (400 pmol) DM025; 16 μL (400 pmol) DM026; and 288 μL PCR supermix. Each reaction was divided into 6 equal aliquots and cycling was performed as described above. The PCR products were pooled, diluted to 400 μL with water, extracted once with phenol-chloroform-isooamyl alcohol (25:24:1 v/v), and precipitated. All of the PCR product was used for in vitro transcription. Transcription, labeling, and gel-purification were performed as described in the section above called “Synthesis, labeling, purification, and quantification of RNA”. The selection process was repeated until the percentage of bound RNA eluted with guanine reached a maximum.

2.8. Cloning and sequencing of cDNAs

PCR products made from selected RNA was cloned using a TA cloning kit (Invitrogen) according to the manufacturer’s instructions. Inserts were sequenced using the M13 reverse primer. Sequencing was performed by Eurofins Genomics. Sequence alignments were performed with Megalign which is part of the Lasergene software package from DNASTar.

3. Results

3.1. Adapting a guanine riboswitch for use as a guanine sensor in vitro

In vitro, guanine binding stabilizes a conformation of the guanine riboswitch that includes a premature transcriptional terminator (Fig. 1A). We attempted to convert the xpt-phaX guanine riboswitch (Mandal et al., 2003) into a sensor by using 3 fragments of the riboswitch as shown in Fig. 1B. We synthesized the first 91 nucleotides of the riboswitch by in vitro transcription and labeled the RNA at its 3' end with fluorescein. This fragment, which we called xpt RNA (1–91), spans the aptamer domain of the riboswitch. The other two fragments were DNA oligonucleotides, called 5'T-1 and 3'T-1, with sequences that corresponded to the 5' and 3' halves of the terminator stem. In addition to pairing with 3'T-1 to form an analog of the terminator, 5'T-1 can pair with the 3' end of xpt RNA (1–91) to form a duplex that mimics the antiterminator (compare Fig. 1A and B). The strategy for our in vitro guanine assay was to label 5'T-1 at its 5' end with a quencher, anneal it to fluorescein-labeled xpt RNA (1–91), and incubate the duplex with guanine and 3'T-1. We anticipated that guanine binding to xpt RNA (1–91) would stabilize the P1 stem resulting in a strand-exchange reaction in which 5'T-1 dissociates from xpt RNA (1–91) and anneals to 3'T-1 (Fig. 1B). Thus, we expected to observe an increase in the fluorescence intensity as the quencher moved away from the fluorescein. Recently, Steinert et al. used a similar system to study the kinetics of this strand-switching reaction (Steinert et al., 2017).

We first used an electrophoretic mobility shift assay (EMSA) to determine whether we could anneal 5'T-1 to xpt RNA (1–91) to form a structure analogous to the antiterminator. We chose to exclude magnesium ions from the reaction to prevent Mg2+-catalyzed hydrolysis of the RNA during the annealing reaction. We found that 5 oligos that form a stable duplex at room temperature even when 5'T-1 was present in 100-fold molar excess (Fig. 2). As a positive control we performed the same experiment using an oligo (called 5'T-2) that could form a 15 base-pair perfect duplex with xpt RNA (1–91). This annealing reaction went to completion in the presence of a 10-fold or 100-fold molar excess of 5'T-2 (Fig. 2). In fact, the reaction with 5'T-2 went to completion even when added to xpt RNA (1–91) in a 1:1 ratio (data not shown). Since the desired duplex with 5'T-1 was unstable in the absence of magnesium ions, we needed to find an oligo that could form a stable, but not too stable, duplex with xpt RNA (1–91) under our chosen conditions. We reasoned that if the duplex was too stable, it would not efficiently undergo the desired strand-exchange reaction. Therefore, we used the EMSA to test a variety of oligos for their ability to anneal to xpt RNA (1–91). We identified 5 oligos that formed a stable duplex at room temperature in the absence of magnesium ions when mixed with xpt RNA (1–91) at a 1:1 M ratio (Fig. 3A). We chose oligonucleotide 5'T-7 for further study because it formed the duplex with the lowest predicted melting temperature.

The sequences of 5'T-1 and 3'T-1 were the same as that found in the natural guanine riboswitch. Since we could not use 5’T-1, we reasoned that we would have to find an alternative to 3'T-1. We used the EMSA to find oligonucleotides that could efficiently compete with xpt RNA (1–91) for pairing with 5'T-7 in the presence, but not in the absence, of guanine. Fig. 3B shows the sequences of the oligos tested. 3'T-3 was the oligo that worked best in this strand-exchange assay. We looked for...
conditions that gave the highest “signal-to-background” ratio. That is, we wanted to minimize the amount of strand-exchange that occurred in the absence of guanine (the “background”) and maximize the amount of strand-exchange that occurred in the presence of guanine (the “signal”). We found the optimal reaction conditions to be as follows: 1:2:10 ratio of xpt RNA (1–91) to 5’T-7 to 3’T-3 in 50 mM Tris pH 7.4 and 100 mM KCl incubated overnight (∼18 h) at 4°C (Fig. 3C). Under these conditions, there was very little background (Fig. 3C, Lane 3) and, in the presence of a 4:1 ratio of guanine to xpt RNA (1–91), the strand-exchange reaction went nearly to completion (Fig. 3C, Lane 5). Importantly, inclusion of 2 mM MgCl2 significantly decreased the signal-to-background ratio due to an increase in the background (data not shown).

Next, we performed the fluorescence-quenching assay. Fig. 4A shows the desired guanine-triggered strand exchange reaction. As expected, the fluorescence intensity increased with guanine concentration and reached a maximum at about 1 μM guanine (Fig. 4C). To assess the ligand specificity of the assay, we compared the signal produced by 5 μM guanine to that produced by 5 μM or 50 μM guanosine, adenine, and hypoxanthine. As expected from previously reported dissociation constants for these ligands (Mandal et al., 2003; Gilbert et al., 2009), our sensor could detect guanosine and hypoxanthine but with reduced sensitivity compared to guanine, and could not detect 50 μM adenine (Fig. 4D).

3.2. Hybrid sensors for the detection of other ligands

We wanted to extend our approach to other riboswitches. However, we did not want to have to re-optimize the sequences of the DNA oligonucleotides and the assay conditions for each new sensor. Therefore, we asked whether we could use hybrid riboswitches composed of the P1 stem from the guanine riboswitch (and some flanking single-stranded RNA) and the ligand-binding domain of a different riboswitch. If so, we could produce sensors for a variety of ligands that allowed us to use the same oligonucleotides (5’T-7 and 3’T-3) and the same conditions as used for the guanine assay.

We attached the ligand-binding domain of a 2'-deoxyguanosine riboswitch from M. florum (Kim et al., 2007) to the P1 stem of the guanine riboswitch and performed fluorescence quenching assays. The design of the sensor is shown in Fig. 5. The results of the assays are shown in Fig. 6. We used the program M-fold (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) to verify that our hybrid construct was likely to adopt the desired secondary structure in the absence of 5’T-7. We were able to use the same assay conditions as used in the guanine assay except we found that this reaction required the presence of magnesium ions. (MgCl2 was added after annealing 5’T-7 to the hybrid sensor to avoid Mg2+-catalyzed RNA hydrolysis at high temperature.) The signal with the hybrid sensor reached a maximum at about 1 μM 2’-deoxyguanosine (Fig. 6A). Although inclusion of 2 mM Mg2+ increased the background, the hybrid sensor was able to reproducibly detect as little as 15 nM 2’-deoxyguanosine (Fig. 6B). The sensor retained the same ligand specificity as the naturally-occurring 2’-deoxyguanosine riboswitch (Kim et al., 2007). In addition to 2’-deoxyguanosine, the sensor could detect guanosine and guanine but with much lower sensitivity (Fig. 6C). The sensor could not detect 50 μM 2’-deoxyadenosine.

Next, we attached the ligand-binding domain of a 3’,5’-cyclic-di-guanylate (c-diGMP) type I riboswitch from V. cholerae (Sudarsan et al., 2008) to a truncated version of the P1 stem of the guanine riboswitch. We found that we had to remove two base-pairs from near the top of the P1 stem in order for the hybrid sensor to fold into the proper secondary structure as predicted by the program M-fold. The design of the hybrid sensor is shown in Fig. 7. The results of the fluorescence-quenching assays are shown in Fig. 8. As with the 2’-deoxyguanosine sensor, the c-diGMP sensor worked only in the presence of magnesium ions. The maximum signal was obtained with ~3 μM c-diGMP (Fig. 8A) and we could reproducibly detect as little as 3 nM c-diGMP (Fig. 8B). Fig. 8C shows the desired guanine-triggered strand exchange reaction. As expected, the signal produced by 5 μM guanine (Fig. 4B). The assay was sensitive as the naturally-occurring 2’-c-diGMP; 2’-5’-c-diAMP; 2’-5’-c-diGMP; 2’-GMP; or 3’-GMP. Fluorescence values are the averages of 3 independent reactions. Error bars are standard deviations. Asterisks indicate signals that are significantly higher than background (P < 0.05).
shows that the sensor is highly specific for c-diGMP. It could not detect 50 μM 3′,5′-c-diAMP, 2′-GMP, or 3′-GMP. The signal produced by c-GAMP (2′,5′-3′,5′-cyclic guanosine monophosphate-adenosine monophosphate) at 1 μM was barely above background. Surprisingly, the signal did not increase with 50 μM c-GAMP. One possible explanation is that c-GAMP binds readily to the sensor but only poorly induces the strand-exchange reaction.

3.3. Strategy for the selection of sensors with novel ligand specificity: proof-of-principle

To further extend the utility of our approach, we devised an in vitro selection scheme to produce sensors with novel ligand specificity. The idea is to partially randomize the sequence of an existing sensor and select variants capable of detecting ligands that are structurally-related to the original ligand. The selection strategy is illustrated in Fig. 9A for a partially-randomized version of our guanine sensor. The partially-randomized pool will contain RNAs with a wide variety of sequences but each RNA will be related to the guanine sensor. The hypothesis is that some of the variants will have altered ligand specificity. The system is identical to the fluorescence quenching assay shown in Fig. 1B except the quencher on 5′T-7 was replaced with a magnetic bead via a biotin-streptavidin interaction, and the sequence of the ligand-binding domain (dotted line) was partially randomized. 3′T-3 is not shown because it was not included in the selection experiment discussed in the text. (B) Alignment of the apt RNA (1–91) cDNA with cDNA sequences selected with guanine. Bases that differ from those found in apt RNA (1–91) are highlighted in black. “N” represents a base that could not be called by the sequencing software which was assumed to be a “T”. Nucleotides 21-75 were partially randomized. (C) Assay of selected RNA function. Using the fluorescence quenching assay, the ability of selected RNAs S6, S8, S11, or S3 to detect 100 nM guanine was compared to that of apt RNA (1–81). The y-axis (Net Fluorescence Intensity) is the difference between the fluorescence intensity produced in the presence of 100 nM guanine minus the background fluorescence produced in the absence of ligand. Values are the averages of 3 independent reactions. Error bars are standard deviations. Unlike S6, S8, and S11 RNAs, the fluorescence intensity produced by S3 RNA was not significantly higher than background.
added 180 pmol of heat-denatured xpt RNA (1–91) to magnetic beads with 200 pmol of bound 5′-T-7. We found that the annealing reaction was very slow. After incubating overnight, only about 30 pmol of RNA was bound to the beads. Longer incubation times did not result in more RNA binding. This result suggested that a large fraction of 5′-T-7 on the beads was inaccessible to the RNA. We then measured the amount of RNA eluted from the beads after incubation for various lengths of time with 5 μM guanine and 150 pmol of 3′-T-3 under the same conditions as used for the quenching assay. We measured the level of background elution by incubating the beads under identical conditions but in the absence of guanine. We found that the highest signal-to-background ratio was achieved with an incubation time of 6 h.

Interestingly, we found that the signal-to-background ratio increased when the experiment was performed without 3′-T-3. This was due to the fact that the amount of RNA eluted with buffer (background) decreased more than the amount of RNA eluted with guanine (signal). 3′-T-3 had the opposite effect on the fluorescence quenching assay (data not shown). The role of 3′-T-3 is to capture 5′-T-7 as it dissociates from xpt RNA (1–91), thus driving the strand exchange reaction in the forward direction. However, since the RNA binds very slowly when 5′-T-7 is on the beads, it appeared that 3′-T-3 was not required to prevent the eluted RNA from re-attaching to the beads during the 6 h incubation. Under the optimal conditions, about 7.5 pmol (25%) of the bound xpt RNA (1–91) eluted with 5 μM guanine. Only about 0.8 pmol (∼2.5%) of RNA eluted when the beads were incubated with assay buffer only, giving a ∼10:1 signal-to-background ratio.

To test our selection scheme, we performed a proof-of-principle experiment. To produce the initial RNA pool, we partially randomized the sequence of the guanine sensor and bound ∼30 pmol (1.8 × 10¹³ molecules) of the RNA to the magnetic beads as described above. We randomized the sequences of only the joining regions (J1-2, J2-3, and J3-1) and the base pairs at the bottom of the P1, P2, and P3 stems (see Fig. 1B). We did not randomize the remainder of the stems or their single-stranded loops. Since xpt RNA (1–91) was only partially randomized, the initial RNA pool included a significant amount of RNA with no sequence changes. Thus, we attempted to isolate the original guanine sensor (and, possibly, functional variants) by using guanine as the ligand during selection. After only 4 rounds of selection, the RNA elution efficiency increased from background levels (∼2% elution) to about 20% elution. The elution efficiency did not increase with further rounds of selection. Since 20% elution is similar to the elution efficiency of pure xpt RNA (1–91) (∼25%), we predicted that our selected RNA pool consisted primarily of xpt RNA (1–91). We sequenced 16 cDNA clones prepared from the selected RNA and found that the sequences of 9 of the clones were identical to that of xpt RNA (1–91) (Fig. 9B). Three of the sequences differed from xpt RNA (1–91) at only 1 or 2 positions (clones S6, S8, and S11 in Fig. 9B). Clone S6 had two sequence changes that converted the A25-U45 pair at the base of stem P2 to a G-C pair. It is known that the structure of the P2 stem is an important determinant of ligand affinity and specificity (Edwards and Bathey, 2009). Clone S8 had a single base change that disrupted the G27-C43 pair near the middle of P2 by changing the G27 pair to a U. Since this base was not changed in the original randomized RNA pool, it probably arose during the selection due to an error made by reverse transcriptase or Taq DNA polymerase. Clone S11 changed U48 to J2-3 to a C. It is known that U48 bulges out of the ligand binding pocket and does not contact the bound guanine (Serganov et al., 2004), so it is not surprising that xpt RNA (1–91) could tolerate this change. The other 4 sequences (clones S3, S9, S16, and S17) contained a large number of changes compared to xpt RNA (1–91) and probably represented RNAs that eluted randomly from the beads (not shown in Fig. 9B). We transcribed the RNAs encoded by clones S6, S8, and S11 and S3, and tested each of them for their ability to detect 100 nM guanine in the fluorescence quenching assay (Fig. 9C). The fluorescence signals produced by both S6 and S8 RNA were about half that produced by xpt RNA (1–91). These results are consistent with the known effects of mutations in the P2 stem of the guanine riboswitch (Edwards and Bathey, 2009). S11 RNA gave the same signal as xpt RNA (1–91). S3, the RNA with a highly divergent sequence could not detect 100 nM guanine.

4. Discussion

Although much work has focused on using riboswitches as biosensors in vivo, less effort has been exerted toward using riboswitches to detect ligands in vitro. One highly successful approach for making sensors for both in vitro and in vivo detection of ligands has been to fuse various aptamer domains via a short “communication module” to a fluorescent aptamer such as “Spinach” (Litke et al., 2016; Paige et al., 2011; Nakayama et al., 2012; Kellenberger et al., 2013; Bhadra and Ellington, 2014; Kellenberger et al., 2015; Ketterer et al., 2016; Bose et al., 2016). The fusions are designed such that ligand binding to the aptamer domain allows the fluorescent aptamer to fold into its active conformation. However, this approach often requires fairly extensive redesign and optimization for each new aptamer domain. Our work sought to convert riboswitches into highly sensitive and specific sensors and to devise a system that would facilitate the isolation of sensors with novel ligand specificities without the need for extensive optimization for each new sensor. This was accomplished by exploiting the modular nature of riboswitches and optimizing the stabilities of the ligand-free and ligand-bound forms of our sensors. We are currently testing whether this approach can be extended to aptamer domains from riboswitches that detect ligands other than purine derivatives and riboswitches that activate rather than inhibit gene expression upon ligand binding. As shown by Ceres et al. designing hybrid “ON” switches poses a particular challenge (Ceres et al., 2013b).

We realized that our approach would not necessarily work for every riboswitch and, of course, nature has not designed riboswitches that can detect every possible ligand of interest. Many groups have attempted to extend the ligand specificity of sensors by replacing naturally-occurring aptamer domains with RNA aptamers produced by in vitro selection using the standard SELEX protocol. This approach has been successful for some applications but has also failed because ligand binding did not induce the required conformational change (Robertson and Ellington, 2000). To circumvent this problem, we and others previously devised an in vitro selection strategy for isolating RNAs that not only bind to the desired ligand but that are also guaranteed to undergo a desired conformational change (Nutiu and Li, 2005; Morse, 2007; Rajendran and Ellington, 2008; Vandenberg and Morse, 2009). Based on our sensor design and our previous selection strategy, we devised a scheme for changing the ligand specificity of naturally-occurring riboswitches.

For our proof-of-principle selection experiment, we partially randomized the ligand-binding domain of the guanine riboswitch and attempted to isolate the original guanine riboswitch using guanine as the ligand. The success of this experiment showed that our strategy works. In addition to isolating the original guanine riboswitch, we found three functional variants, indicating that our selection scheme will also be useful for structure-function studies. We sequenced only 16 of the RNAs selected with guanine, but deep sequencing should reveal most, if not all, of the possible functional variants.

Importantly, each of our hybrid sensors represent a new starting point for in vitro selection and there will be no need to extensively optimize the conditions for each new selection. Thus, we should be able to find sensors for ligands related to the cognate ligand of each riboswitch, and we will be able to perform studies that probe the sequence and structure requirements for each new ligand-binding domain.

It is instructive to compare our selection scheme to recently reported experiments. Koizumi et al. completely randomized the aptamer domain of a self-cleaving allosteric hammerhead ribozyme and selected novel aptazymes that could detect cGMP, cAMP, and cCMP (Koizumi et al., 1999). Schemes for selecting functional self-cleaving ribozymes have an intrinsic advantage over selecting riboswitches. Functional
Riboswitches are being applied as analytical tools in biochemistry, genetics, cell biology, medicine, environmental science, forensics, and many other areas (Lee et al., 2016; Machtel et al., 2016). In the exciting new field of synthetic biology, riboswitches have become the preferred tool for constructing new gene regulatory devices and genetic circuits (Groher and Suess, 2014; and Jo and Shin, 2009; Topp et al., 2010; Weber and Fussenegger, 2011; Chappell et al., 2015). Our work will contribute to increasing the availability of riboswitches with novel ligand specificity.

Funding

This work was supported by the Defense Threat Reduction Agency (MIPR HDTRA1620511 to D.M.); the Office of Naval Research; and the Department of the U.S. Naval Academy.

Acknowledgements

Thanks to Shirley Lin and Ina O’Carroll for critical reading of the manuscript.

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